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The profile of phenolic compounds by HPLC-MS in Spanish oak (*Quercus*) honeydew honey and their relationships with color and antioxidant activity

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ABSTRACT

Due to the importance of the authentication of the botanical and geographical origin of honey, this document is focused on the use of phenols as potential chemical markers in an attempt to distinguish and characterize one of the non-floral honey types, oak (*Quercus* sp.) honey, from other honeydew honeys produced in other regions. The development and validation of ultra-high-performance liquid chromatography with mass spectrometry detection enables the identification of 23 phenolic compounds in 58 Spanish oak honeydew honey samples. The proposed compounds, above all salicylic acid (mean = 24.31 mg 100 g^{-1}), but also *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, naringenin, and galangin could further contribute as a helpful tool in the quality control of this non-floral honey, and therefore distinguish it from European honeydew honey and those from other regions. Furthermore, many positive correlations between phenolic compounds, CIELAB color parameters, and antioxidant activity are established. This research presents a complete phenolic composition and the largest sampling of one of the most appreciated honeydew honey varieties. This is also one of the singular studies that have successfully linked individual phenolic acids and flavonoids with physicochemical parameters within the same unifloral honey.

1. Introduction

Phenolic compounds are secondary metabolites of plants. They represent one of the most important groups of compounds that occur in plants. Flavonoids and phenolic acids (derivatives of benzoic and cinnamic acids) are the most important class of phenols present in honey (Anklam, 1998). Phenols come to honey through flower nectar or honeydew, as well as via propolis and pollen. Honey is rich in phenolic compounds, which act as natural antioxidants and are becoming increasingly popular in human diets because of their potential role in the treatment of various diseases and their contribution to human health (Alvarez-Suarez, Gasparrini, Forbes-Hernández, Mazzoni, & Giampieri, 2014).

Honey can commonly be divided into two categories in accordance with its botanical source: flower or honeydew honey. Floral honey comes from the nectar while honeydew honey originates essentially from plant exudations or excretions produced by insects. Honeydew honey could be derived by sacking insects (Binazzi & Scheurer, 2009; Carter & Maslen, 1982), by plant secretions as a result of damage by insects, or through high phloem pressure which is common in many Spanish oak forests where *Quercus* (*Q. ilex* and *Q. pyrenaica*) trees display high quantities of phloem sap in their acorns (Fig. 1).

Currently, the market interest in honeydew honeys is growing due to their stronger medicinal, antibacterial, and antioxidant characteristics, and consumers display greater interest in non-floral honeys (honeydew) than in floral (nectar) honeys (Castro-Vázquez, Díaz-Maroto, & Pérez-Coello, 2006; Kocyigit et al., 2019; Pita-Calvo & Vázquez, 2018; Silva et al., 2020; Stanciu et al., 2008). Many studies have demonstrated distinctions in the chemical content between nectar and honeydew honeys (Bentabol Manzanares, Hernández-García, Rodríguez-Galdón, Rodríguez-Rodríguez, & Díaz-Romero, 2011; Pita-Calvo & Vázquez, 2017; Recklies, Peukert, Kölling-Speer, & Speer, 2021; Simova,

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Atanassov, Shishiniova, & Bankova, 2012), and several studies have revealed higher antioxidant activities in honeydew honey than in floral honeys (Karabagias et al., 2018; Lachman, Orsák, Hejtmánková, & Kovářová, 2010; Vela, de Lorenzo, & Perez, 2007). The application of phenol studies in the recognition and acknowledgment of honey was recommended as an instrument for judging the source of honey, since the HPLC coupled to UV-detector remains the main method of choice for the analysis of these constituents on honeys (Valverde, Ares, Elmore, & Bernal, 2022). Many chemical compounds were proposed as markers of numerous types of honeydew honey: *trans*-oak lactone and quercitol for *Quercus* honey; tridecane and 1-chlorooctane for *Pinus* honey; and 3-carene as a differentiate compound between pine honeys from Turkey and Greece (Castro-Vázquez et al., 2006; Pita-Calvo & Vázquez, 2017; Sanz, Gonzalez, De Lorenzo, Sanz, & Martínez-Castro, 2005; Tananaki, Thrasyvoulou, Giraudel, & Montury, 2007). Generally, a diversity of phenol compounds were identified in many European honeydew honeys, such as caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, sinapic acid, vanillic acid, acacetin, apigenin, catechin, chrysin, epicatechin, hesperidin, kaempferol, luteolin, naringenin, pinocembrin, quercetin, rutin, and taxifolin (Nešovićet al., 2020; Silici, Sarioglu, & Karaman, 2013; Vasić et al., 2019).

Despite the fact that oak (*Quercus* sp.) honeydew honeys are commonly consumed in Spain, research into the identification of this non-floral honey type remains limited. In recent years, however, efforts have been made regarding palynological and common physicochemical parameters (Flores, Escuredo, & Seijo, 2015; Jara-Palacios et al., 2019; Seijo, Escuredo, & Rodríguez-Flores, 2019; Terrab, Berjano, Sanchez, Gómez Pajuelo, & Díez, 2019), although research into phenolic compounds remains scant (Vazquez, Armada, Celeiro, Dagnac, & Llompart, 2021).

Spain is the largest honey producer in the EU, and in terms of quality it is the primary producer (36,000 tons) and exporter (22,000 tons) within the EU. A wide variety of unifloral honeys are produced in this country, with oak honeydew honey being the third most important (6011 tons; García de Frutos, 2020). In order to avoid honey adulteration and fraud, knowledge of a more analytical nature regarding this honey type is crucial for the consumers and food industry due to the limitations in the characterization of honeydew honey by melissopalynological assessment (Seijo et al., 2019; Terrab et al., 2019). The identification of markers could therefore help in its authentication and increase its commercial value. To this end, the principal aim of our study involves establishing suitable phenolic compounds for the certification and characterization of oak honeydew honey. An HPLC method, previously validated on honeys by Hernanz et al. (2022), was therefore employed for the assessment of profile phenolic compounds on one of the most appreciated non-floral honeys: oak honeydew honey. Another objective of this research involved testing the relationship between the individual phenols and chromatic parameters and antioxidant activity (ABTS and TBARS assays), using a multivariate statistical technique, such as Multiple Linear Regressions (MLR).

2. Materials and methods

2.1. Honey samples

This research was carried out on 58 oak (*Quercus* sp.) honey samples collected in 2014 in diverse Spanish regions where the oak trees are

Fig. 1. *Quercus ilex* acorns showing honeydew secretions. a. Dried acorn (left) and developing acorn secreting honeydew, that arise between the cupule and the nut of the acorn. b. Developing acorn secreting honeydew. c. Dried acorn secreting honeydew. d. Cupule with honeydew after the fall of the nut. Photographs: P. López (a), and A. Gómez Pajuelo (b–d).

dominant. The samples were provided directly by apicultural associations or by local beekeepers and were immediately stored at 4–5 °C. The samples have been authenticated by the beekeepers as oak honeys. All the analyses (pollen, physicochemical, and those performed in the present study) were carried out between 2015 and 2016 on the same 58 honey samples. The results of pollen analysis have already been published by Terrab et al. (2019) as well as those analyses of the different physicochemical parameters (such as color, mineral content, sugar profile, and antioxidant activity) (Jara-Palacios et al., 2019).

2.2. Phenolic compounds

2.2.1. Standards and reagents

The chemical and chromatographic solvents (acetonitrile, ethyl acetate, formic acid, and methanol) were of analytical and HPLC grade. Water was purified in a Nanopure®DiamondTM system (Barnsted Inc. Dubuque, IO). Caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, salicylic acid, sinapic acid, syringic acid, vanillic acid, apigenin, catechin, chrysin, epicatechin, galangin, hesperidin, isorhamnetin, kaempferol, luteolin, naringenin, pinocembrin, and quercetin were all purchased from Sigma Aldrich (Madrid, Spain).

2.2.2. Extraction procedure

The extraction has been carried out following Gheldof, Wang, and Engeseth (2002). Honey (1 g) was dissolved in 3 mL of acidified water (pH 2) and filtered through a column of Amberlite XAD-2 SPE (300 mg; 20–60 mesh size, SUPELCO-Sigma-Aldrich, USA), which was previously conditioned with 3 mL of methanol, ultrapure water, and acidified water (pH 2). The column was washed with 3 mL of water and acidified water (pH 2) to remove sugar and polar compounds, and phenolic compounds were eluted with 3 mL of methanol. This extract was concentrated to dryness under reduced pressure. The residue obtained was dissolved in 500 μL of water and extracted with ethyl acetate (500 μL x 3). Organic extracts were combined and then concentrated. The dried residue was dissolved in 200 μL of 0.01% formic acid to be analyzed by UHPLC subsequent to filtration through a hydrophilic PVDF Millex-HV 0.45 μm syringe filter (Millipore, Bedford, MA, USA).

2.2.3. Identification of phenolic compounds

Identification of the phenolic compounds was performed using Agilent 1200 series HPLC equipment (Agilent, USA) coupled to a 6410 triple quadrupole (QqQ) mass spectrometer (MS) equipped with an electrospray ionization source (Agilent, USA). Separation of the analytes was performed using a HALO C18 (50 × 4.6 mm i. d.; 2.7 μm) analytical column (Teknokroma, Spain) protected by a HALO C18 (5 × 4.6 mm i. d.; 2.7 μm) guard column (Teknokroma, Spain). Analytes were separated by gradient elution using acetonitrile (containing 0.1% formic acid) (solvent A) and water (0.1% formic acid) (solvent B) as its mobile phase at a flow rate of 0.6mLmin^{-1}. Column temperature was maintained at 35 °C. The elution program was as follows: 0–5min, isocratic 5% of solvent A; 5–20min, linear gradient from 5 to 50% of solvent A; 20–22min, isocratic 50% of solvent A; 22–24min, linear gradient from 50 to 100% of solvent A; and, finally, back to initial conditions (5% of solvent A) for 2min. Ionization was carried out using the following settings: MS capillary voltage, 3000 V; drying gas flow rate, 9Lmin^{-1}; drying gas temperature, 350 °C; and nebulizer pressure, 40 psi. Instrument control and data acquisition were carried out with Mass Hunter software (Agilent, USA). Detection was performed in multiple reaction-monitoring mode (MRM). MS/MS parameters were optimized by injection, without column, of 10mgL^{-1} individual standard solutions of target compounds using water (0.1% formic acid) and acetonitrile (0.1% formic acid) in mobile phase. Both positive and negative ionization modes were monitored. Due to their abundance, two transitions were selected for each compound. Both transitions and the relation between their abundance were utilized for the identification of target compounds.

2.2.4. Quantification of phenolic compounds

Quantitative analyses were performed by UHPLC in an Agilent 1290 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode-array detector (DAD). The scan was set from 200 to 770 nm, and a C18 Eclipse Plus 120 column (1.8 μm, 50 × 2.1 mm). The quantification was carried out by external calibration from the areas of the chromatographic peaks obtained at 280 nm. The applied method was previously validated by Hernanz et al. (2022). Linearity of the method was evaluated through the consideration of the detector response (area units) to different amounts of phenolics by means of linear regression. Stock solutions of phenolic standards were prepared in acetonitrile at a concentration of 100mgmL^{-1}. The calibration curves were made up of six dilutions of the stock solutions in 0.01% formic acid. The limit of detection (LOD) and that of quantification (LOQ) were calculated following Hernanz et al. (2022).

2.3. Color parameters

Color was assessed by tristimulus from the colorimetry based on reflectance spectra, using a CAS-140 B spectroradiometer. The following CIELAB parameters were determined: L^* (lightness), a^* and b^* (two color coordinates), h_{ab} (hue angle) and C^*_{ab} (chroma). Results of color parameters are presented in Table S3.

2.4. Antioxidant activity

The ability to scavenge the $ABTS^{\bullet+}$ radical was measured in vitro based on the ABTS assay. 50 μL of honey sample was added to 2 mL of the $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm. Results were expressed as Trolox equivalent antioxidant capacity (TEAC), considered as the μmol of Trolox with the same antioxidant capacity as 100 g of honey (μmoL TE 100 g^{-1}).

The lipid peroxidation inhibition was determined by the TBARS assay. Livers of rats were mixed with 100 μL of honey sample, 25 μL of 20 mM cumene hydroperoxide (oxidant compound), Tris-HCl buffer, 10% trichloroacetic acid at 4 °C and 1 mL of 2-thiobarbituric acid. The TBARS were measured by determining absorbance at 535 nm. Results are expressed as percentage of inhibition of lipid peroxidation (% inhibition). Results of antioxidant activity are presented in Table S3.

2.5. Statistical analysis

Statistical v.8.0 software was employed for the various statistical procedures. Simple and multiple regressions were performed, and the statistically significant level was taken into account at $P < 0.05$. Multiple Linear Regressions (MLR) were carried out to detect relationships between individual phenolic compounds, and chromatic parameters and antioxidant activity (ABTS and TBARS assays), as previously published by Jara-Palacios et al. (2019).

3. Results and discussion

3.1. Analysis of phenolic compounds

The applied method permits the identification of 23 phenolic compounds, corresponding to three groups: (a) seven benzoic acids; (b) four hydroxycinnamic acids; and (c) 12 flavonoids. In this way, 16 phenolic compounds (see Fig. 2) were quantified: (a) hydroxybenzoic acids (gallic, *p*-hydroxybenzoic, protocatechuic, salicylic, syringic, and vanillic acids); (b) hydroxycinnamic acids (caffeic, chlorogenic, *p*-coumaric, and ferulic acids); and (c) flavonoids (apigenin, chrysin, galangin, luteolin, naringenin, and pinocembrin). Moreover, seven phenolic compounds (sinapic acid, catechin, epicatechin, hesperidin, isorhamnetin, kaempferol, and quercetin) were identified but not

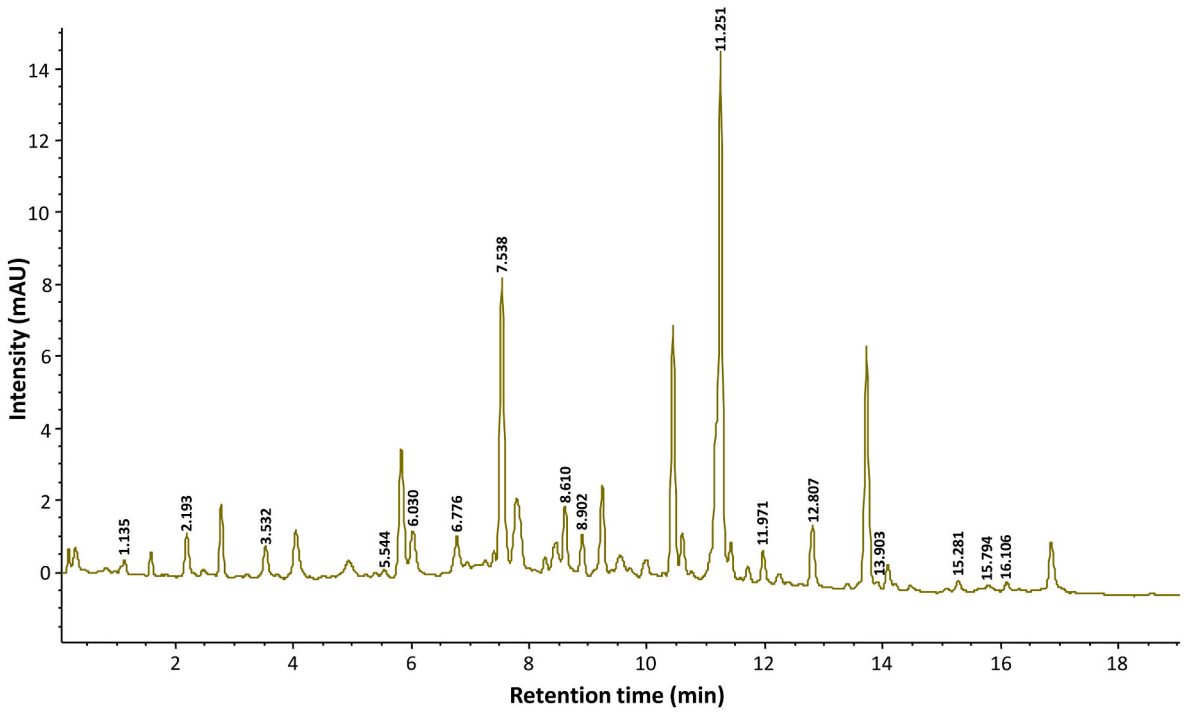

Fig. 2. Chromatogram of a honey sample (nº 5) at 280 nm. 1. Gallic acid (tr: 1.135min), 2. Protocatechuic acid (tr: 2.193min), 3. *p*-hydroxybenzoic acid (tr: 3.532min), 4. Vanillic acid (tr: 5.544min), 5. Caffeic acid (tr: 6.030min), 6. Syringic acid (tr: 6.776min), 7. *p*-coumaric acid (tr: 7.538min), 8. Ferulic acid (tr: 8.610min), 9. Chlorogenic acid (tr: 8.902min), 10. Salicylic acid (tr: 11.251min), 11. Luteolin (tr: 11.971min), 12. Naringenin (tr: 12.807min), 13. Apigenin (tr: 13.903min), 14. Galangin (tr: 15.281min), 15. Chrysin (tr: 15.794min), 16. Pinocembrin (tr: 16.106min).

quantified, since their concentration in the honey samples waslower than the LOD (see Table S1).

In Table 1, the 16 phenolic quantified compounds can be observed. The phenolic profile was characterized by the presence of such salicylic (0.01–65.47 mg 100 g^{-1}), gallic (nd-0.136 mg 100 g^{-1}), protocatechuic (nd-0.435 mg 100 g^{-1}), *p*-hydroxybenzoic (0.0651–6.115 mg 100 g^{-1}), vanillic (nd-0.095 mg 100 g^{-1}), syringic (0.086–4.086 mg 100 g^{-1}), caffeic (nd-0.484 mg 100 g^{-1}), *p*-coumaric (nd-2.723 mg 100 g^{-1}), ferulic (nd-0.244 mg 100 g^{-1}), and chlorogenic (nd-6.635 mg 100 g^{-1}). The most abundant compound was salicylic acid present in 57 of the 58 samples with a mean of 24.31 mg 100 g^{-1}, which represents 83.36% of the total amount of all quantified phenolic compounds. The phenolic acids present in most samples were *p*-hydroxybenzoic (present in 100% of the samples; mean = 0.803 mg 100 g^{-1}), syringic (present in 100% of the samples; mean = 0.740 mg 100 g^{-1}), *p*-coumaric (present in 98% of the samples; mean = 0.594 mg 100 g^{-1}), protocatechuic (present in 93% of the samples; mean = 0.195mg 100 g^{-1}), and caffeic acids (present in 97% of the samples; mean = 0.176 mg 100 g^{-1}). Other acids present in most of the honey samples (93%), but in low quantities, was ferulic acid (mean = 0.071 mg 100 g^{-1}), whereas, vanillic, gallic, and chlorogenic acids were more infrequent in the samples (present in 53, 34, and 9% of the samples, respectively). Regarding the flavonoids, three compounds were present in most of the samples with relative intermediate concentrations: pinocembrin (present in 93% of the samples; mean = 0.383 mg 100 g^{-1}), naringenin (present in 95% of the samples; mean = 0.362 mg 100 g^{-1}), and galangin (present in 97% of the samples; mean = 0.145 mg 100 g^{-1}). The remaining flavonoids were detected only in minor samples, with concentrations ranging from 0.454 mg 100 g^{-1} for luteolin (present in 16% of the samples) to 0.077 mg 100 g^{-1} for chrysin (present in 53% of the samples) (for more details see Table S2).

Table 1
Phenolic acids, flavonoids and phenols contents of the 58 *Quercus* honeydew honey samples analyzed (mg 100 g^{-1}).

	Mean	SD[a]	Min	Max
Gallic acid	0.021	0.033	nd	0.136
Protocatechuic acid	0.195	0.094	nd	0.435
p-hydroxybenzoic acid	0.803	0.954	0.065	6.115
Vanillic acid	0.031	0.031	nd	0.095
Syringic acid	0.740	0.683	0.086	4.086
Salicylic acid	24.31	12.74	0.001	65.47
Σ Hydroxybenzoic acids[b]	26.10	13.09	2.567	67.65
Caffeic acid	0.176	0.124	nd	0.484
Ferulic acid	0.071	0.055	nd	0.244
p-coumaric acid	0.594	0.526	nd	2.723
Chlorogenic acid	0.499	1.645	nd	6.635
Σ Hydroxycinnamic acids[b]	1.340	1.907	0.114	8.714
Σ Phenolic acids[b]	27.44	13.52	3.330	68.57
Naringenin	0.362	0.162	nd	0.733
Galangin	0.145	0.075	nd	0.376
Chrysin	0.077	0.075	nd	0.249
Pinocembrin	0.383	0.203	nd	1.144
Luteolin	0.454	1.078	nd	3.875
Apigenin	0.302	0.644	nd	3.214
Σ Flavonoids[b]	1.723	1.575	0.497	8.836
Σ Phenols total[b]	29.16	13.50	4.015	70.80

[a] SD: Standard deviation. Nd: not detected.
[b] Σ Hydroxybenzoic acids; Σ hydroxycinnamic acids; Σ Phenolic acids; Σ Flavonoids; Σ Phenols total: sum of all individual hydroxybenzoic acids, hydroxycinnamic acids, phenolic acids, flavonoids, and phenolic compounds, respectively.

Salicylic acid was the compound with the highest amounts of all the phenolic acids (see Fig. 3; mean = 24.31 mg 100 g^{-1}), this compound has been suggested as a biomarker for honeydew honey (Daher & GülaCàçar, 2008). The concentration of this hydroxybenzoic acid was much higher of any other studies made in honeydew honeys. Thus, salicylic acid was quantified in Brazilian *Mimosa scabrella* honeydew honeys but in exceptionally low quantities (<1 mg 100 g^{-1}) and in very few samples (Azevedo et al., 2021; Silva et al., 2020; Silva, Gonzaga, Fett, & Costa, 2019). Likewise, salicylic acid has either been quantified in many European honeydew honeys (Croatian *Salix* honeydew honey, Greek fir, pine and oak honeys, Czech honeydew honey, and Slovak forest honey) in very low amounts (generally <1 mg 100 g^{-1})

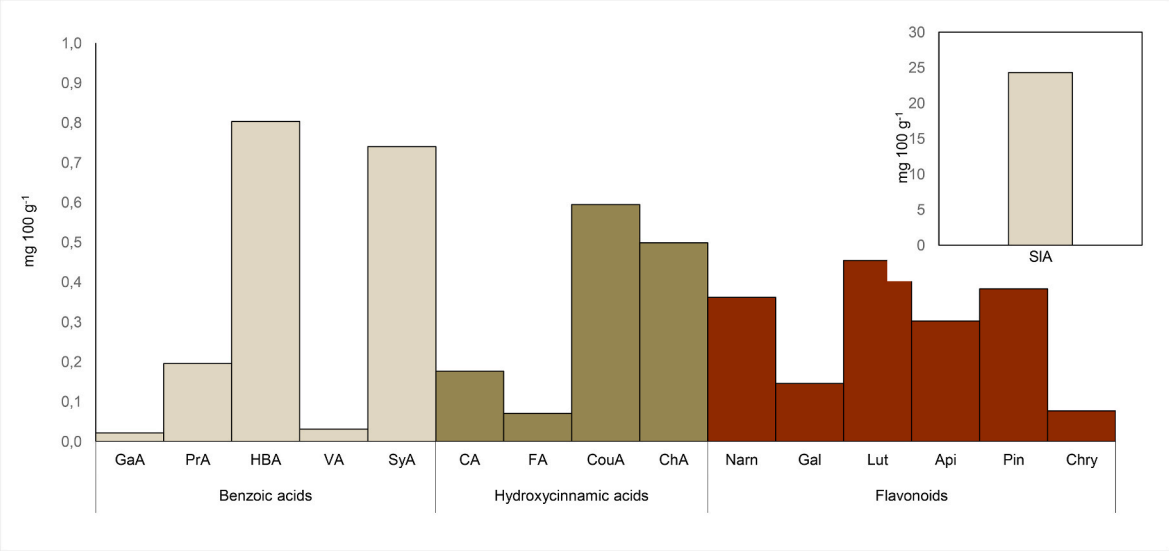

Fig. 3. Distributions of the main amounts of the 16 phenolic compounds quantified in oak *Quercus* honeydew honey by phenol groups. GaA – Gallic acid; PrA – Protocatechuic acid; HBA – *p*-Hydroxybenzoic acid; VA – Vanillic acid; CA – Caffeic acid; FA – Ferulic acid; SyA – Syringic acid; CouA – *p*-coumaric acid; ChA– Chlorogenic acid; SlA- Salicylic acid; Narn – Naringenin; Gal – Galangin; Chry – Chrysin; Pin – Pinocembrin; Lut – Luteolin; Api – Apigenin.

(Halouzka, Tarkowski, & Zeljković, 2016; Koulis et al., 2022; Kováčik, Grúz, Biba, & Hedbavny, 2016; Tuberoso, Jerković, Bifulco, & Marijanović, 2011), or has not been detected at all, as in Turkish oak and pine honeys (Can et al., 2015; Silici et al., 2013).

In several honeydew honeys, *p*-hydroxybenzoic acid has been found but generally in lower amounts (e.g., Nešović et al. (2020) quantified *p*-hydroxybenzoic acid in Montenegrin honeydew honeys with mean = 0.210 mg 100 g^{-1}). Vasić et al. (2019) detected this acid in different types of Croatian honeydew honeys, but always in very low quantities ranging from mean = 0.035 mg 100 g^{-1} in Silver fir honeys to mean = 0.045 mg 100 g^{-1} in Evergreen oak honey; likewise,Halouzka et al. (2016) detected this acid in Czech honeydew honey (mean = 0.292 mg 100 g−1), and Chessum, Chen, Hamid, and Kam (2022) in New Zealand honeydew honeys (mean = 0.051 mg 100 g^{-1}), while Can et al. (2015) found higher quantities in three Turkish oak honeys (mean = 5.02 mg 100 g^{-1}) and three pine honeydew honey samples (mean = 2.92 mg 100 g^{-1}). Other authors have not detected or quantified the *p*-hydroxybenzoic acid in the honeydew honey analyzed (Azevedo et al., 2021).

Syringic acid was quantified in many floral honeys albeit in small concentrations, ranging from <0.07 mg 100 g^{-1} in acacia and buckwheat honeys (Biesaga & Pyrzynska, 2013; Dimitrova, Gevrenova, & Anklam, 2007) to 172 mg 100 g^{-1} in jujube and manuka honeys (Stephens et al., 2010; Wabaidur et al., 2015). In honeydew honeys, the syringic acid was quantified in very low amounts ranging from 0.004 to 0.303 mg 100 g^{-1} in Turkish pine and oak honeys (Can et al., 2015; Haroun, Poyrazoglu, Konar, & Arti, 2012), from 0.014 to 0.132 mg 100 g^{-1} in Brazilian bracatinga honeydew honey (Azevedo et al., 2021), from 0.014 to 0.025 mg 100 g^{-1} in Czech honeydew honey (Halouzka et al., 2016), while in Polish honeydew honey, it has been found at levels of 0.05 mg 100 g^{-1} (Socha et al., 2011). On the other hand, syringic acid was not quantified in any type (*Abies*, *Quercus*, *Acer,* or conifers) of Croatian honeydew honeys (Vasić et al., 2019), nor in New Zealand honeydew honeys (Chessum et al., 2022), nor in Montenegrin honeydew honeys (Nešović et al., 2020).

With respect to p-coumaric acid, this was not detected in Polish pine honeydew honey (Stanek & Jasicka-Misiak, 2018), on the other hand, it was quantified in low concentrations (mean < 0.090 mg 100 g^{-1}) in different types of Turkish and Croatian honeydew honeys (Can et al., 2015; Vasić et al., 2019), and also in Italian honeydew honeys with mean = 0.025 mg 100 g^{-1} Pichichero, Canuti, & Canini, 2009). However, it was found in concentrations similar to those found in our study ranging from 0.333 in Czech honeydew honey to 0.534 mg 100 g^{-1} in Polish honeydew honey (Socha et al., 2011; Halouzka et al., 2016), and from 0.027 to 0.350 mg 100 g^{-1} in Galician honeydew honeys (Northwest Spain) Armada, Celeiro, Dagnac, & Llompart, 2021), whereby the Romanian honeydew honey presents the highest amounts (mean = 0.910 mg 100 g^{-1}; Oroian & Sorina, 2017).

Another hydroxycinnamic acid found in most of samples (mean = 0.195 mg 100 g^{-1}) was that of protocatechuic acid (3,4-dihydroxybenzoic acid), whose presence enables the honeydew honey to be distinguished from floral honeys (Recklies et al., 2021), and is also proposed as a likely biomarker of honeydew honey (Pita-Calvo & Vázquez, 2018). Protocatechuic acid was quantified in lower quantities in Montenegrin honeydew honeys (range = 0.061–0.152 mg 100 g^{-1}; Nešović et al., 2020), and also in several types of Croatian honeydew honeys (Vasić et al., 2021), ranging from 0.004 mg 100 g^{-1} in Hungarian oak honey to 0.029 mg 100 g^{-1} in Montpellier maple honey. Similar contents of those found in our samples were found in Galician honeydew honey (range = 0.330–1.0 mg 100 g^{-1}; Armada, Celeiro, Dagnac, & Llompart, 2021), in Polish fir and Czech honeydew honeys (mean = 0.440 and 0.605 mg 100 g^{-1}, respectively; Halouzka et al., 2016; Kuś, Jerković, Marijanović, & Tuberoso, 2017), and in Brazilian bracatinga honeys (range = 0.097–0.228 mg 100 g^{-1}; Azevedo et al., 2021). On the other hand, contrasting quantities for this compound were found in Turkish honeydew honey: while the highest values (range = 8–74 mg 100 g^{-1}) have been detected by Can et al. (2015), other authors have found similar content levels to those found in our study (range = 0.163–0.598 mg 100 g^{-1}; Haroun et al., 2012).

In relation to the flavonoids, three compounds were present in most of the samples with relatively intermediate concentrations (naringenin, pinocembrin, and galangin) (see Fig. 3). In our oak honey, naringenin was quantified with a mean of 0.362 mg 100 g^{-1}. This flavanone was found in certain unifloral honeys (acacia, buckwheat, and jujube) but only in small quantities of <0.05 mg 100 g^{-1} (Biesaga & Pyrzynska, 2013; Wabaidur et al., 2015), except for Moroccan *Euphorbia resinifera* where Hernanz et al. (2022) found amounts similar to those encountered in our samples (mean = 0.448 mg 100 g^{-1}). In honeydew honeys, Vasić et al. (2019) quantified this flavanone in very low concentrations in various Croatian honeydew honey types (mean < 0.007 mg 100 g^{-1}), with similar results found for honeydew honeys that were Polish (mean = 0.098 mg 100 g^{-1}; Socha et al., 2011), Czech (range = nd-0.003 mg 100 g^{-1}; Halouzka et al., 2016), Slovenian (range = nd-0.016 mg 100

g^{-1}; Bertoncelj, Polak, Kropf, Korošec, & Golob, 2011), and Montenegrin (range = 0.001–0.047 mg 100 g^{-1}; Nešović et al., 2020). With respect to pinocembrin, this was found in most of the samples with a mean of 0.383 mg 100 g^{-1}, while the majority of studies carried out on Brazilian, Turkish, Polish, and Greek honeydew honeys have either failed to detect or have quantified this flavanone in very low concentrations (<0.05 mg 100 g^{-1}) (Bertoncelj et al., 2011; Can et al., 2015; Haroun et al., 2012; Koulis et al., 2022; Seraglio et al., 2016; Silici et al., 2013; Silva et al., 2019; Socha et al., 2011). However, honeydew honey from the Czech Republic, Montenegro, Romania, and New Zealand contained an amount of pinocembrin (ranging from 0.129 to 0.666 mg 100 g^{-1}) similar to those found in our study (Chessum et al., 2022; Ciucure & Geană, 2019; Halouzka et al., 2016; Nešović et al., 2020). Lastly, the galangin was quantified in most of the samples with a mean of 0.454 mg 100 g^{-1}. The amounts found in our research were much higher than those in other honeydew honeys, most of which presented either undetectable/unquantifiable levels of this flavonol (Can et al., 2015; Chessum et al., 2022; Halouzka et al., 2016; Seraglio et al., 2016; Silva et al., 2019; Socha et al., 2011), or found low to very low levels of galangin ranging from 0.01 to 0.032 mg 100 g^{-1} (Bertoncelj et al., 2011; Koulis et al., 2022; Pichichero et al., 2009; Vasić et al., 2019).

Generally, the results obtained in different studies of honeydew honey mention a major diversity in phenol compounds; however, in several studies the authors considered only the honey samples as honeydew, without specifying the exact source of the honeys. Furthermore, the vast majority of the authors analyzed only a few honey samples, fewer than three, and only a few reached 10 samples, while an extremely limited number included up to 20 samples (see Pita-Calvo & Vázquez, 2018; Seraglio et al., 2019). According to the data obtained from the 23 phenolic compounds identified, and the 16 phenolic compounds quantified in the 58 samples analyzed, it can be stated that Spanish oak honeydew honeys revealed that the most distinguished compounds were salicylic acid, syringic acid, naringenin and galangin, which were present in more than 95% of the samples; on the other hand, and due to the higher concentration found in these four compounds, our honeydew honeys could be perfectly differentiated from those of other regions (Central Europe, Greece, Turkey, Brazil, and New Zealand).

3.2. Statistical analysis

3.2.1. Phenolic compounds vs. color

Color is one of the most useful parameters for the characterization of honey; it can vary from white to black. Many authors have correlated color of honey with the content of phenolic and flavonoids, whereby the darkest honeys present the highest values of phenolic content. The relationships between phenolic compounds and color parameters were explored by means of MLR whereby each phenolic compound is considered as a dependent variable, and the L^*, a^*, b^*, and L^*, h_{ab}, C^*_{ab} as predictors (see Table S4). High correlations ($P < 0.05$) were obtained between gallic acid and (L^*, a^*, b^*) and (L^*, h_{ab}, C^*_{ab}) ($R^2 = 0.383$ and 0.439, respectively), between naringenin and (L^*, a^*, b^*) and (L^*, h_{ab}, C^*_{ab}) ($R^2 = 0.392$ and 0.432, respectively), and between luteolin and (L^*, h_{ab}, C^*_{ab}) ($R^2 = 0.400$). Studies on honeys that have related phenolic contents and color are numerous, many of which were carried out correlating total phenolic and flavonoid contents and the Pfund colorimetric method, but very few correlated the chromatic attributes with the individual phenols (Hernanz et al., 2022). If we focus on the studies carried out on dark honeys, higher phenolic and flavonoid contents were observed by Al-Farsi, Al-Amri, Al-Hadhrami, and Al-Belushi (2018). Similarly, greater phenolic concentrations were found in dark brown *vs.* light yellow honeys from Kosovo (Daci-Ajvazi, Mehmeti, Zeneli, & Daci, 2017). In studies carried out on Central and South American honeys, many authors have observed a high correlation between dark honeys and flavonoid content (Mexico: Balcázar-Cruz et al., 2019; Argentina: Cabrera, Perez, Gallez, Andrada, & Balbarrey, 2017; Brazil: Pontis, Costa, Silva, & Flach, 2014). Finally, correlations between total phenolic and flavonoid contents and CIELAB color attributes have been carried out by Escuredo, Rodríguez-Flores, Rojo-Martínez, and Seijo (2019) on Galician Spanish honeys, and have revealed close correlations between total phenol and flavonoid content and the chromatic CIELAB parameters (a^*, b^*, L^*, C^*_{ab}, and hue).

3.2.2. Phenolic compounds vs. antioxidant activity

Various studies have recognized phenolic compounds constitute the main contents responsible for the antioxidant activity of honey (Cianciosi et al., 2018; Rice-Evans & Miller, 1996). The relationships between phenolic compounds and antioxidant activity, measured through ABTS and TBARS assays, were explored using MLR (see Table S5). Significant relationships ($P < 0.05$) were found between gallic acid and ABTS ($R^2 = 0.288$), protocatechuic acid and TBARS ($R^2 = 0.349$), and between *p*-coumaric acid and TBARS ($R^2 = 0.295$). Many authors established a major relationship between phenol content and the antioxidant activity of honey, thereby indicating that phenols are responsible for the scavenging activity and are mainly attributed to flavonoid compounds (Alvarez-Suarez, Tulipani, Romandini, Vidal, & Battino, 2009; Becerril-Sánchez, Quintero-Salazar, Dublán-García, & Escalona-Buendía, 2021; Gheldof et al., 2002; Rababah et al., 2014).

4. Conclusions

Due to the limitations of pollen analysis assessment in the authentication of honeydew honey, other analytical methods were suggested for a more accurate identification of honey sources. This research constitutes a valued contribution for the phenolic characterization of one of the most important honeydew honey types. This research presents a complete phenolic composition and sampling of Spanish oak (*Quercus*) honeydew honeys. In this study, UHPLC-MS analysis leads to the quantification of 16 phenolic compounds with the highest amounts found for salicylic acid, furthermore, syringic acid, naringenin, and galangin could be considered as the most distinguished compounds of this non-floral honey due to their presence in most of the samples and to their high concentrations when they are compared with honeydew honey from other regions (Central Europe, Greece, Turkey, Brazil, and New Zealand). Moreover, positive correlations were found not only between phenolic compounds and chromatic parameters but also between phenolic compounds and antioxidant activity. Finally, and despite the effort made studying various botanical and physicochemical parameters (pollen, carbohydrates, minerals, color, phenols, etc.), the study of the volatile composition, would help even more to the perfect differentiation and characterization of this non-floral honey type.

CRediT authorship contribution statement

Dolores Hernanz: Conceptualization, Supervision, Writing – original draft, Funding acquisition. **M. José Jara-Palacios:** Investigation, Writing – review & editing. **Juan Luis Santos:** Investigation, Conceptualization. **Antonio Gómez Pajuelo:** Conceptualization, Supervision. **Francisco J. Heredia:** Conceptualization, Supervision, Writing – review & editing. **Anass Terrab:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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